Amendments to the Specification:

Please amend the paragraph beginning on page 12, line 6, as follows:

In order to test the hypothesis that a tubercle bacillus isolated from cattle (now known as *M. bovis*) could transmit pulmonary tuberculosis following oral administration, Drs. Calmette and Guerin developed a medium containing beef bile that enabled the preparation of fine homogenous bacillary suspensions (Calmette and Guerin, 1905). An *M. bovis* strain obtained from Dr. Norcard, was passaged every 21 days in this medium and after the 39th passage, the strain was found to be unable to kill experimental ~~animal~~ animals (Gheorghiu, 1996). "Between 1908 and 1921, the strain showed no reversion to virulence after 230 passages on bile potato medium" (Id.), which is consistent with the attenuating mutation being a deletion mutation. In the animal studies that followed, the strain ('BCG') was found to be attenuated but it also protected animals receiving a lethal challenge of virulent tubercle bacilli (Calmette and Guerin, 1920). BCG was first used as a vaccine against tuberculosis in 1921. From 1921 to 1927, BCG was shown to have protective efficacy against TB in a study on children (Weill-Halle and Turpin, 1925; Calmette and Plotz, 1929) and adopted by the League of Nations in 1928 for widespread use in the prevention of tuberculosis. By the 1950's after a series of clinical trials, the WHO was encouraging widespread use of BCG vaccine throughout the world (Fine and Rodrigues, 1990). Although an estimated 3 billion doses have been used to vaccinate the human population against tuberculosis, the mechanism that causes BCG's attenuation remains unknown.

Please amend the paragraph beginning on page 13, line 18, as follows:

It is also worth noting that in the study of Chambers *et al.* (2000), both BCG and the BCG mutants seemed to protect better against *M. bovis* challenge than *M. tuberculosis*. If we assume the reverse correlate is true, we could hypothesize that optimal immunity against *M. tuberculosis* could be achieved with a *M. tuberculosis*-derived mutant that grew in the mammalian host.

Please amend the paragraph beginning on page 14, line 17, as follows:

In other embodiments, the invention is directed to the use of an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex for the manufacture of a medicament for treatment of a mammal ~~that is~~ that does not have severe combined immune deficiency but is deficient in $CD8^+$ lymphocytes . In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

Please amend the paragraph beginning on page 15, line 9, as follows:

FIG. 3a-3g ~~is~~ are photographs, micrographs and autoradiographs showing that the *M. tuberculosis* H37Rv Δ*RD1* mutant exhibits two distinct colonial morphotypes. Panel a, *M. tuberculosis* H37Rv. Panel b, *M. tuberculosis* H37Rv Δ*RD1*. Panel c, *M. tuberculosis* H37Rv Δ*RD1* ::2F9. Panel d, Southern analysis of *M. tuberculosis* H37Rv Δ*RD1* *Nco*I-digested genomic DNA, isolated from three smooth and three rough colonies and probed with DFS. Panels e–g, Colonial morphotypes at higher magnification. e, Smooth morphotype at week 4. f, Rough morphotype at week 4. g, Rough morphotype at week 6.

Please amend the paragraph beginning on page 15, line 16, as follows:

FIG. 4a-4d ~~is~~ are graphs showing the growth kinetics of *M. tuberculosis* H37Rv Δ*RD1* in BALB/c mice. Mice were infected with $2X10^6$ CFU through tail injection. Time to death was noted and at day 1, week 4, 8, 14, and 22 post-infection, mice were sacrificed to determine the mycobacterial burden in the spleen, liver, and lung. The numbers represent the means of CFUs in organs derived from three animals. The error bars represent the standard errors of the means. Panel a, Time to death assay in BALB/c mice. Panel b, Spleen. Panel c, Liver. Panel d, Lung.

Please amend the paragraph beginning on page 15, line 23, as follows:

FIG. 5a-5i ~~is~~ are micrographs from pathological studies of infected BALB/c mice. Panels a – c, Lungs from mice infected with $2x10^6$ CFU of *M. tuberculosis* H37Rv examined at 4, 8 and 14 weeks post-infection. The mild to moderate pneumonia at 4 and 8 weeks (a and b) progressed to severe consolidating granulomatous pneumonia at 14 weeks post infection (c). Panels d – f, Lungs from mice infected with $2x10^6$ CFU of *M. tuberculosis* H37Rv Δ*RD1* examined at 4, 8 and 22 weeks post-infection showing moderate pneumonia at 8 weeks post-infection (e) and persistent bronchitis and multifocal pneumonitis at 22 weeks post-infection (f). Panels (g)– (i), Mild lung lesions from mice infected with $2x10^6$ CFU of BCG at 4, 8 and 22 weeks post-infection. Mild focal granulomas scattered widely in the lung at each time point with predominately lymphocytic accumulations in foci at 22 weeks post-infection.

Please amend the paragraph beginning on page 16, line 20, as follows:

FIG. 8A-8B ~~shows~~ show graphs summarizing experiments demonstrating the attenuation, limited replication and persistence of Δ*nadBC* mutant in immunocompetent mice. Panels A and B, Bacterial loads in lungs and spleen of C57BL/6 mice infected with wild type *M. tuberculosis* H37Rv (•) or Δ*nadBC* mutant (○). Mice were infected intravenously with 10^6 CFU of each strain. CFUs were assayed at various time points on 7H11 agar with or without nicotinamide supplementation where required. The results represent means ± standard errors of four to five mice per group. Panel C, Survival of C57BL/6 mice (n=12 per group) infected with 10^6 CFU of wild-type bacteria ~~(○)~~ (•) or 10^6 CFU of Δ*nadBC* mutant (○).

Please amend the paragraph beginning on page 17, line 6, as follows:

FIG. 10A-10F ~~shows~~ show graphs summarizing experiments demonstrating that pantothenate auxotrophy leads to attenuation of Δ*panCD* mutant in mice. ~~a.~~ A. Survival of BALB/c SCID mice (n = 12 per group) infected intravenously with H37Rv (○) or panCD-complemented strain (•) or Δ*panCD* mutant (▲) or M. bovis BCG-P (□). ~~b.~~ B. Bacterial numbers in the spleen (○), liver (□) and lung (Δ) of SCID mice infected intravenously with H37Rv or the bacterial numbers in the spleen (•), liver (■) and lung (▲) of mice infected with Δ*panCD* mutant. ~~c.~~ C. Survival of immunocompetent BALB/c mice (n = 16 per group) infected with H37Rv (○) or panCD-complemented strain (•) or Δ*panCD* mutant (▲). ~~d, e, f.~~ D, E, F. Bacterial numbers in lung (~~d~~ D), spleen (~~e~~ E) and liver (~~f~~ F) of immunocompetent BALB/c mice infected intravenously with either H37Rv (○), *panCD*-complemented strain (•) or Δ*panCD* mutant (▲). Data are means ± standard errors of four to five mice per group.

Please amend the paragraph beginning on page 17, line 17, as follows:

FIG. 11A-11F ~~shows~~ show micrographs and graphs summarizing experiments demonstrating that the Δ*panCD* mutant produces less tissue pathology in lungs of infected BALB/c mice and protects mice against challenge with virulent *M. tuberculosis*. ~~a.~~ A. Severe consolidating granulomatous pneumonia (★) obliterating the normal lung parenchyma at 3 weeks post-infection with H37Rv. ~~b.~~ B. Severe consolidating granulomatous pneumonia (★) obliterating the normal lung parenchyma at 3 weeks post-infection with the *panCD*-complemented strain, similar to the wild type strain. ~~c.~~ C. Mild lung infection caused by the Δ*panCD* mutant at 3 weeks post-infection. Localized multifocal granulomas (arrows) scattered widely in the lung. Most of the lung is normal alveolar spaces and airways. ~~d.~~ D. Lung of mouse infected with Δ*panCD* mutant examined histologically at 23 weeks post-infection. Occasional focal, mild perivascular and interstitial infiltrations composed of predominately lymphocytes (arrows). Most of the lung is normal alveolar spaces and airways. ~~e, f.~~ E, F. The attenuated Δ*panCD* mutant protects mice against aerogenic challenge with virulent *M. tuberculosis* Erdman. Subcutaneously immunized mice were challenged after 90 days through the aerosol route. The CFU numbers reflect the bacterial burden at 28 days post aerosol challenge in the lung (e E) and spleen (~~f~~ F). Naive mice - black fill; mice infected with 1 dose *panCD* - light shade; mice infected with 2 doses *panCD* - dark shade; mice infected with BCG-P - unshaded.

Please amend the paragraph beginning on page 18, line 20, as follows:

FIG. 14A-14C ~~is~~ are graphs summarizing experiments demonstrating the clearance of the lysine auxotroph in SCID mice. The viable bacterial counts are shown for the spleens, livers, and lungs of SCID mice injected intravenously with the lysine

auxotroph strain and the prototrophic control strain. Three mice were assayed at each time point. The error bars indicate the standard deviations of the mean values. Note that the counts at time zero are the counts obtained at 24 hours post-injection, as described in Example 5. Panels A, B and C show the log of the viable bacteria in each organ after injection with $1x10^7$ CFU of the *Lys*$^-$ *M. tuberculosis* mutant mc^{2}3026 (□), or $1x10^7$ CFU of the complemented *Lys*$^+$ *M. tuberculosis* strain mc^{2}3026/pYUB651 ([[□]]◆).

Please amend the paragraph beginning on page 18, line 29, as follows:

FIG. 15A-15C ~~is~~ are graphs summarizing experimental results of experiments that establish the vaccine efficacy of the *M. tuberculosis* lysine auxotroph mc^{2}3026. C57Bl/6 mice were injected intravenously with $1x10^6$ CFU of the *M. tuberculosis* lysine auxotroph mc^{2}3026, followed by one or two additional injections at 4 week intervals. Five mice were sacrificed weekly after each immunization and the viable bacteria counts of the auxotroph determined in the lungs and spleens. Control mice were given a similar amount of BCG-Pasteur or only PBST. Shown in Panel A is the clearance of the auxotroph from the lungs of the mice after each immunization period; one injection (■), two injections ([[◆]] ◇), and three injections ([[•]] ○). Three months after the initial immunization the vaccinated and control mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route. Five challenge mice were sacrificed following the challenge period and the lung homogenates plated to check the viable counts of the challenge inoculum. Groups of vaccinated and control mice were sacrificed at 14, 28, and 42 days later and the lung and spleen homogenates plated to determine viable colony forming units. Shown in Panel B are the viable challenge bacteria per lung of mice given one dose of the M. tuberculosis lysine auxotroph, and in panel C,

the viable challenge bacteria per lung of mice given two doses of the auxotroph. Key: Viable challenge bacteria per lung of mice given the *M. tuberculosis* lysine auxotroph $mc^2$3026 (■), BCG-Pasteur (◇), or PBST (○). P values are indicated in the figure. Note that the results shown here are for the lungs. Similar results (not shown) were obtained from the spleens in all the experiments.

Please amend the paragraph beginning on page 20, line 11, as follows:

FIG. 22A-22I ~~is~~ are graphs and micrographs of experimental results showing that $mc^2$6030 is severely attenuated in immunocompromised mice. ~~a,~~ A. Survival of SCID mice infected intravenously with 10^2 CFUs of H37Rv (○) or 10^5 CFUs of $mc^2$6030 (•). ~~b-c,~~ B, C. Bacterial numbers in the spleen (~~b~~ B), lungs (~~c~~ C), and of H37Rv (○) or $mc^2$6030 (•) infected SCID mice. The results represent means ±standard errors of four to five mice per group. ~~d,~~ D. Survival of gamma interferon gene-disrupted (GKO) C57BL/6 mice (n = 10 mice) infected intravenously with 10^5 CFUs of H37Rv (○) or $mc^2$6030 mutant (•) or M. bovis BCG-P (▲). ~~e,~~ E. Survival of immunocompetent C57BL/6 mice (n = 10 mice) infected intravenously with 106 CFUs of H37Rv (○) or $mc^2$6030 (•). ~~f-g,~~ F, G. Bacterial numbers in spleen (~~g~~ F) and lungs (~~h~~ G) of C57BL/6 mice infected intravenously with H37Rv (○) or $mc^2$6030 (•). The results represent means ±standard errors of four to five mice per group. ~~h,~~ H. Mild perivascular, lymphocytic infiltrates caused by strain ~~mc26030~~ $mc^2$6030 in C57BL/6 mice at 3 weeks post-infection. ~~i,~~ I. Severe granulomatous pneumonia in the lungs of C57BL/6 mice infected with H37Rv at 3 weeks post-infection.

Please amend the paragraph beginning on page 20, line 25, as follows:

FIG. 23A-23E ~~is~~ are graphs of experimental results showing that vaccination with ~~mc26030~~ $mc^2$6030 induces both short-term and long-term protection in C57BL/6 mice. ~~a-b,~~ A, B. Immunocompetent C57BL/6 mice were immunized subcutaneously (s.c) with $mc^2$6030 or BCG-P and challenged with virulent *M. tuberculosis* Erdman through the aerosol route at 3 months after the initial immunization. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs and spleen of infected mice. ~~c-d,~~ C, D. Immunocompetent C57BL/6 mice were immunized subcutaneously (s.c) with $mc^2$6030 or BCG-P and challenged with virulent *M. tuberculosis* Erdman through the aerosol route at 8 months after the initial immunization. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs and spleen of infected mice. The results represent means ±standard errors of five mice per group. **P < 0.01, ***P < 0.001 indicate statistical differences between the experimental and unvaccinated control groups. ~~e,~~ E. Survival of immunocompetent C57BL/6 mice (n = 10 mice) immunized subcutaneously with a single dose of $mc^2$6030 (•) or BCG-P (▲) and challenged 3 months later with virulent M. tuberculosis Erdman through the aerosol route. Unvaccinated mice served as naive controls (○).

Please amend the paragraph beginning on page 21, line 7, as follows:

FIG. 24A-24F ~~is~~ are graphs of experimental results showing that vaccination with $mc^2$6030 protects and confers greater survival advantage to $CD4^{-/-}$ mice from tuberculous challenge. ~~a-b,~~ A, B. Protection induced by a single dose of $mc^2$6030 in CD4-deficient mice following aerosol challenge with virulent *M. tuberculosis* Erdman. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs (a) and spleen (b) from 5 mice per group. **P< 0.001

indicate statistical differences between the experimental and unvaccinated control groups. ~~c,~~ C. Survival of $CD4^{-/-}$ mice (n = 5 or 6 mice) immunized subcutaneously with a single dose of $mc^2$6030 (•) or BCG-P (▲) and challenged 3 months later with virulent M. tuberculosis Erdman through the aerosol route. Unvaccinated mice served as naive controls (○). ~~d-e,~~ D, E. Treatment of $mc^2$6030-vaccinated $CD4^{-/-}$ mice with anti-CD8 antibody does not abolish the protection seen in $mc^2$6030-vaccinated control antibody treated $CD4^{-/-}$ mice. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs (~~d~~ D) and spleen (e E) from 5 mice per group. **P< 0.001 indicate statistical differences between the experimental and unvaccinated control groups. ~~f,~~ F. Survival of vaccinated GKO mice following an aerosol challenge with virulent *M. tuberculosis*.

Please amend the paragraph beginning on page 21, line 22, as follows:

Fig. 25A-25F ~~is~~ are micrographs of experimental results showing that. $mc^2$6030 vaccinated $CD4^{-/-}$ mice display improved lung pathology following challenge with virulent M. tuberculosis. ~~a,~~ A. Severe pneumonia in lung of unvaccinated mice at 28 days post-aerosol challenge, with ~~d~~ D, large numbers of *M. tuberculosis* Erdman organisms demonstrated by acid-fast stain. ~~b,~~ B. Lung from mouse vaccinate with $mc^2$6030 showing milder multifocal areas of pneumonia composed of macrophages and numerous lymphocytes, with e E, lower number of *M. tuberculosis* Erdman organisms indicating protection following immunization. ~~c,~~ C. BCG vaccinated mouse. Similar localized areas of pneumonia adjacent to the airways post-aerosol challenge and ~~f~~ F, reduced numbers of acid-fast organisms similar to $mc^2$6030 vaccinated mice.

Please amend the paragraph beginning on page 43, line 15, as follows:

Table 3. The attenuated *M. tuberculosis* Δ*nadBC* and Δ*panCD* mutants protect against aerogenic challenge with *M. tuberculosis* Erdman. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously either once or twice (6 weeks apart) with 10^6 CFUs of mutant strains. Control mice were vaccinated subcutaneously with 10^6 CFUs of BCG-Pasteur. Three months after the initial immunization with either Δ*nadBC or* Δ*panCD* mutant or BCG, the mice were aerogenically challenged with approximately 100 CFUs of acriflavin-resistant *M. tuberculosis* Erdman (Ac^rMTB) strain as described earlier (Collins, 1985) After 28 days, the challenged mice were sacrificed, and the lungs and spleens of individual mice were removed aseptically and homogenized separately in 5 ml of Tween 80-saline using a Seward stomacher 80 blender (Tekmar, Cincinnati, OH). The homogenates were diluted serially in Tween 80 saline and plated on Middlebrook 7H11 agar with or without appropriate supplements as required. Samples from the BCG-vaccinated controls were plated on 7H11 agar containing 2 mg of thiophenecarboxylic acid hydrazide (Sigma Chemical Co., St Louis, MO) per ml to inhibit growth of any residual BCG. The CFU results were evaluated using the one-way ANOVA analysis of the Graph Pad InStat program. The numbers in ~~paranthesis~~ parenthesis represent the differences between naïve and vaccinated organ CFUs.

Please amend the paragraph beginning on page 47, line 6, as follows:

We constructed a double deletion mutant of *M. tuberculosis* in the *panC* and *panD* genes that are involved in the de novo biosynthesis of pantothenate (FIG. 9b,c). The Δ*panCD* mutant was found to be auxotrophic for pantothenate with no

detectable reversion to prototrophy when $1x10^{10}$ cells were plated on minimal medium. The growth rate of the mutant was identical to wild type H37Rv in broth cultures in the presence of exogenous pantothenate (data not shown). The attenuation of the Δ*panCD* mutant was assessed by infection of immunocompromised SCID mice. SCID mice infected intravenously with H37Rv succumbed to the resulting infection in about 5 weeks. In contrast, all mice infected with the Δ*panCD* mutant survived for more than 36 weeks (average, 253 days) (FIG. ~~10a~~ 10A). This attenuation is due to pantothenate auxotrophy as the full virulence phenotype was restored when the *panCD* wild type genes were integrated into the chromosome of the Δ*panCD* mutant in single copy. Enumeration of bacterial burdens in SCID mice infected with H37Rv and the Δ*panCD*-complemented strain showed a rapid increase in bacterial numbers in the spleen, liver and lung, until they succumbed to infection. In contrast, mice infected with the Δ*panCD* mutant showed an initial drop in bacterial numbers in the spleen and liver followed by a gradual increase in the number of viable bacteria, reaching $1x10^{6}$ colony-forming units (CFU) by day 224 (FIG. ~~10b~~ 10B). Notably, the CFU values increased to $1x10^{8}$ in the lungs of the infected mice. The ability of Δ*panCD*-infected SCID mice to survive despite a substantial bacterial burden in their lungs emphasizes the extent of attenuation in this mutant and compares with the phenotype observed with the *M. tuberculosis whiB3* and *sigH* mutants described recently (Steyn et al., 2000; Kaushal, 2000). Notably, SCID mice infected with bacille Calmette-Guerin-Pasteur (BCG-P) strain succumbed to infection by 83 days (Weber et al., 2000) in contrast to the prolonged survival observed in Δ*panCD*-infected mice.

Please amend the paragraph beginning on page 47, line 29, as follows:

Studies in immunocompetent mice further demonstrate the attenuation of the Δ*panCD* mutant. Survival studies showed that BALB/c mice infected with H37Rv succumbed to infection by day 25 (average, 21 days) and mice infected with an identical dose of the *panCD*-complemented strain succumbed to infection between days 21 to 53 (average, 37 days). Importantly, all mice infected with $1x10^6$ CFU of the Δ*panCD* mutant survived 375 days, when the experiment was terminated (FIG. ~~10e~~ 10C). At 3 weeks post infection, in contrast to the H37Rv strain, BALB/c mice infected with Δ*panCD* mutant showed a 10-fold increase in bacterial numbers in the lungs followed by a gradual decline in viable numbers over the next 38 weeks of infection (FIG. ~~10d~~ 10D) and the bacterial burden gradually declined in the spleen and liver throughout the course of infection (FIG. ~~10e~~ 10E). Histopathologic examination of the lungs from mice infected with either H37Rv or the Δ*panCD* -complemented strain, showed severe, diffuse lobar granulomatous pneumonia (FIG. ~~11a,b~~ 11A,B). The pneumonia affected more than 50% of the lung, and was pyogranulomatous with marked necrosis in the advanced consolidated areas, particularly in the lungs of mice challenged with H37Rv. Both of these strains caused severe granulomatous splenitis and widespread granulomatous hepatitis. At 3 weeks post-infection with the Δ*panCD* mutant, low to moderate numbers of focal infiltrates of mononuclear cells scattered throughout the lung were seen (FIG. ~~11c~~ 11C). The spleen was moderately enlarged with scattered granulomas. Similarly, the liver showed numerous focal granulomas. At 24 weeks post-infection, consistent with the bacterial numbers, histological examination of the lungs from mice infected with the Δ*panCD* mutant showed only occasional focal mild infiltrations, predominately lymphocytic (FIG. ~~11d~~ 11D). The spleen showed only mild histiocytic hyperplasia and there were fewer, focal, predominately lymphocytic accumulations in the liver.

Please amend the paragraph beginning on page 49, line 1, as follows:

As a test of vaccine potential, immunized mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route (Collins, 1985). Following subcutaneous immunization, the Δ*panCD* mutant could not be detected in the spleens or lungs of mice at 8 and 12 weeks. In the naive controls, the bacterial CFU values increased 10,000-fold in the lung during the first month after challenge. Similarly, substantial dissemination and growth in the spleen was detected within one month of the challenge in naive controls. In contrast, mice immunized with single or double doses of the Δ*panCD* mutant displayed statistically significant reductions ($P < 0.05$) in lung and spleen CFU values relative to naive controls. Mice vaccinated with BCG showed similar reduction in organ bacterial burdens compared to the nonimmunized controls (FIG. ~~11e,f~~ 11E,F). In these aerogenic challenge studies, no significant differences were detected in the lung and spleen CFU values for mice vaccinated with either the Δ*panCD* mutant strain or with BCG. At 28 days after the aerogenic challenge with virulent *M. tuberculosis*, histopathological examination of lungs of Δ*panCD* immunized mice revealed a less severe infection relative to the unvaccinated control mice. In controls, severe bronchitis, moderate pneumonia, and spread of the infection to the adjacent lung parenchyma was observed. By comparison, the Δ*panCD* vaccinated mice had milder bronchitis and smaller areas of mild interstitial pneumonitis, with localized areas of granulomatous pneumonia in some mice. Importantly, no lung pathology was detected in vaccinated mice at the time of challenge (data not shown). Two groups of mice that were vaccinated with one or two doses of the Δ*panCD* mutant and then challenged with *M. tuberculosis* Erdman were active and healthy for more than one year following the virulent challenge. Histopathological analysis of lung sections from these mice showed only mild inflammation and fibrosis despite the chronic infection.

Please amend the paragraph beginning on page 53, line 1, as follows:

While infection with BCG and *M. tuberculosis* Δ*RD1* yielded similar survival results in BALB/c mice, there were substantial differences in the growth kinetics in mice. BCG grew in a limited fashion in lungs, liver and spleen in BALB/c mice during the 22 weeks of the experiment (FIG. ~~4B-D~~ 4b-d). In contrast, the *M. tuberculosis* Δ*RD1* strain grew in a fashion indistinguishable from the parental *M. tuberculosis* H37Rv in all mouse organs for the first 8 weeks. Thereafter, mice infected with the parental *M. tuberculosis* failed to contain the infection leading to mortality. Strikingly, mice infected with the *M. tuberculosis* Δ*RD1* showed a definite control over infection resulting in significantly prolonged survival of mice (FIG. ~~4B-D~~ 4b-d).

Please amend the paragraph beginning on page 53, line 10, as follows:

Histopathological examination further demonstrated that the mutant was attenuated in virulence compared to the parent strain H37Rv (FIG. ~~5D-F~~ 5d-f). In contrast to the rapidly progressive infection with the parent strain, the lung lesions caused by the mutant were maximal in mice examined at 8 weeks post-infection. Consolidating granulomatous pneumonia involved an estimated 25-30% of the lung in these mice. Numerous organisms were demonstrated by acid fast staining. The pneumonia subsequently underwent partial resolution. By 14 weeks, and again, at 22 weeks post-infection, the lungs showed peribronchial and perivascular inflammatory cell accumulations and focal, generally non- confluent, granulomas now with a prominent lymphocytes infiltration. The numbers of acid fast bacilli were reduced. Liver lesions consisted of low numbers of scattered granulomas. Spleens were smaller, with persistent granulomas in the red pulp. Mice infected with *M. bovis* BCG showed mild lesions in the lung, liver and spleen

at all time points (FIG. ~~5G-I~~ 5g-i). At longer time intervals post-infection the lesions were fewer in number, and smaller with prominent lymphocytic infiltrations. At 14 weeks post-infection, *M. bovis* BCG was below the level of detection by acid fast staining. In summary, whereas *M. tuberculosis* Δ*RD1* initially grew in a manner similar to the parental *M. tuberculosis* H37Rv, this *RD1* mutant was limited in the extent of spread of infection, particularly in the lung. This contrasts the extensive and severe damage caused by the parent strain. The subsequent resolving granulomas, localization of the lesions and changes in the composition of the inflammatory cell infiltrations suggested that the mice mounted an effective immune response to combat *M. tuberculosis* Δ*RD1* infection and thereby reduced the numbers of viable organisms.

Please amend the paragraph beginning on page 53, line 10, as follows:

While frozen stocks of the original BCG strain do not exist, written records do exist describing the early BCG strains, as Dr. Calmette sent the strains to many laboratories. In a study published in 1929, Petroff and colleagues reported that BCG displayed two distinct colony types (Petroff et al., 1929). One morphotype was a smooth (S) phenotype that was flat and corded (like the parental virulent strain) and the second was a rough and raised (R) phenotype. The *M. tuberculosis* Δ*RD1* mutant was generated independently four times and consistently yielded a 20 to 50% mixture of two colonial morphotypes on Middlebrook medium without Tween 80 (FIG. ~~3B~~ 3b). The distinction of these two types of morphology could be noted even when the colonies were less than two weeks old, as the rough colonies were constricted and elevated with only a small portion of the base of the colony attached to the agar, while the smooth colonies tended to be flattened and spread out. When colonies grew older, e.g. 6 weeks old, the rough colonies remained more constricted compared to those of smooth colonies. The rough

colonies exhibited large folds on the surface (FIG. ~~3F-G~~ 3f-g), as compared to those of the smooth colonies that exhibited small wrinkles (FIG. ~~3E~~ 3e).

Please amend the paragraph beginning on page 54, line 13, as follows:

The generation of two distinct colonial morphotypes must be a phenotypic change induced by the deletion of *RD1*. The morphotypes could not be cloned, as repeated subculturing of either the R or S colonies continued to yield both colonial morphotypes. Moreover, Southern analysis of R and S colonies revealed each morphotype had the same *RD1*-deleted genotype (FIG. ~~3D~~ 3d). Furthermore, complementation of *M. tuberculosis* Δ*RD1* with the *RD1* region restored the mutant phenotype back to the homogenous parental S phenotype (FIG. ~~3A-C~~ 3a-c). These results suggest that the variable morphotypes resulted directly from the *RD1* deletion. It can therefore be postulated that a regulator of colonial morphology is affected by one or more of the deleted genes.

Please amend the paragraph beginning on page 68, line 21, as follows:

An important pre-requisite for any live attenuated vaccine is their safe use even in immunodeficient hosts. To assess the attenuation of this mutant, severe combined immunodeficient (SCID) mice, a highly stringent model for safety, were infected intravenously with H37Rv or mc^{2}6030. Mice infected with 10^2 CFU of *M. tuberculosis* H37Rv strain died within 4 weeks post-infection. Interestingly, 60% of SCID mice infected with 10^5 CFU of mc^{2}6030 survived for over 350 days (FIG. ~~22a~~ 22A). Mice infected with H37Rv showed a rapid increase in bacterial numbers in the lungs, spleen and liver by 3 weeks. In contrast, the bacterial numbers in the spleen of mc^{2}6030-infected mice remained relatively constant

throughout the course of infection (FIG. ~~22b~~ 22B). Bacterial numbers in the lungs of mc^{2}6030-infected SCID mice showed a decrease in the first 3 weeks of infection, but gradually increased to reach 10^8 CFUs by 350 days (FIG. ~~22c~~ 22C). The bacterial titers were constant in the liver throughout the course of infection except for a sharp decline at 3 weeks (data not shown).

Please amend the paragraph beginning on page 69, line 15, as follows:

mc^{2}6030 undergoes limited replication in mice. To evaluate the effect of the multiple mutations in strain mc^{2}6030 on bacterial growth in vivo, immunocompetent BALB/c or C57BL/6 mice were infected intravenously. All BALB/c mice infected with 10^5 CFU of H37Rv succumbed to the resulting infection by 168 days (MST = 134 days). In contrast, all mice infected with 10^5 CFU of mc^{2}6030 survived for over 400 days (data not shown). Similarly, C57BL/6 mice infected with 10^6 CFU of H37Rv succumbed to infection by 260 days (MST = 196 days). All mice infected with 10^6 CFUs of mc^{2}6030 survived over 400 days post-infection (FIG. ~~22e~~ 22E). Bacteriological culture results generally showed a decline in the numbers of H37Rv and ~~mc26030~~ mc^{2}6030 organisms in the spleen (FIG. ~~22f~~ 22F) and liver (data not shown) of C57BL/6 mice after infection. Interestingly, the pulmonary bacterial CFU numbers for both the virulent H37Rv strain and the attenuated mutant reached a constant level at 3 months post-infection (FIG. ~~22g~~ 22G). Importantly, the H37Rv CFU values were at least 100-fold higher than the mc^{2}6030 CFUs in all organs tested at 200 days after the infection.

Please amend the paragraph beginning on page 69, line 28, as follows:

Histopathological examination of organs from infected mice confirmed the marked attenuation of the deletion mutant. At 3 weeks post-infection, an intravenous injection of mc^{2}6030 (FIG. ~~22h~~ 22H) had caused only rare, mild perivascular, lymphocytic infiltrates. The spleens were slightly enlarged with mononuclear cell infiltration in the red pulp. Also, multifocal, mild infiltrations of macrophages and neutrophils were seen in the liver and no acid-fast bacilli were detected. This contrasted with the severe pneumonia (FIG. ~~22i~~ 22I), markedly enlarged spleens, severe diffuse granulomatous hepatitis, and the overwhelming bacterial burden seen in mice infected with the H37Rv strain.

Please amend the paragraph beginning on page 70, line 3, as follows:

mc^{2}6030 induces short and long-term protection in immunocompetent mice. Having assessed the safety and growth kinetics of mc^{2}6030 in both immunodeficient and immunocompetent mice, we evaluated the protective immune responses induced by this attenuated strain. As a test of its vaccine potential, C57BL/6 mice were immunized subcutaneously with 10^6 CFUs of mc^{2}6030 and then were challenged 3 months later with a low dose of virulent *M. tuberculosis* Erdman by the aerosol route. Following subcutaneous immunization, the immunizing mc^{2}6030 mutant bacteria could not be cultured from the spleens or lungs of mice at 8 and 12 weeks postvaccination. At 28 days post-aerosol challenge, mice that were immunized 3 months earlier with a single dose of mc^{2}6030 showed a significant reduction in the lung ($P < 0.01$) and spleen ($P < 0.01$) bacterial CFU values as compared to naïve mice. Consistent with published results, mice vaccinated with 10^6 CFUs of BCG showed similar CFU reductions in the lungs ($P < 0.001$) and spleen ($P < 0.001$) as compared to the naïve controls (FIG. ~~23a,b~~ 23A,B).

Please amend the paragraph beginning on page 70, line 16, as follows:

In order to assess the duration and persistence of the memory immune response, vaccinated and control mice were challenged through the aerosol route 8 months after a single dose vaccination. In the naïve controls at four weeks post-challenge, the bacterial numbers increased dramatically in the lung and substantial dissemination and growth in the spleen were also observed. Strikingly, mice vaccinated with a single dose of mc^{2}6030 or BCG-P displayed statistically significant reduction in bacterial numbers in the lungs ($P < 0.001$) and spleen ($P < 0.001$) relative to naïve controls (FIG. ~~23e,d~~ 23C,D). Our data clearly demonstrate that a single dose of mc^{2}6030 induces a potent and long-lasting protective immune response that is effective in controlling a virulent *M. tuberculosis* challenge in the lungs and spleen of mice even after 8 months following the primary immunization.

Please amend the paragraph beginning on page 71, line 3, as follows:

~~mc26030~~ mc^{2}6030 protects CD4-deficient mice significantly better than BCG against anaerosolized TB challenge. Tuberculosis remains the largest attributable cause of death in HIV-infected individuals (Whalen et al., 2000). HIV infection leads to a loss of $CD4^+$ T cells and previous studies have demonstrated that mice deficient in $CD4^+$ T cells are highly susceptible to *M. bovis* BCG (Ladel et al., 1995) and *M. tuberculosis* infection (Mogues et al., 2001; Caruso et al., 1999). Since BCG vaccination is contraindicated in HIV-infected individuals, we wanted to test if the more attenuated strain, mc^{2}6030, could protect CD4-deficient mice from experimental tuberculosis. CD4-deficient mice were vaccinated subcutaneously with a single dose of 10^6 CFUs of either mc^{2}6030 or BCG-P and

then aerogenically challenged with 100-200 CFUs of *M. tuberculosis* Erdman three months later. At 28 days post-aerosol challenge, the bacterial burden in the lungs ($P < 0.001$) and spleen ($P < 0.001$) of the $mc^2$6030 and BCG-P vaccinated mice was decreased by greater than 99% (>2 $\log_{10}$ CFU) relative to naïve controls (FIG. ~~24a,b~~ 24A,B).

Please amend the paragraph beginning on page 71, line 16, as follows:

To further evaluate the long-lived protection induced by $mc^2$6030, $CD4^{-/-}$ mice were vaccinated, challenged 3 months later with virulent *M. tuberculosis* Erdman through the aerosol route, and followed for survival. All of the naïve mice died within 29 days (MST = 27 ±2 days) of the low dose tuberculous aerogenic challenge (FIG. ~~24c~~ 24C). Strikingly, the mean survival time for the $CD4^{-/-}$ mice vaccinated with a single dose of $mc^2$6030 was 214 ±18 days, a nearly eight-fold extension of the survival period compared to naïves. In contrast, the BCG vaccinated mice survived 158 ± 23 days. The 56-day extension of the MST for the $mc^2$6030-immunized mice relative to BCG vaccinated animals represented a significantly improved outcome ($P < 0.05$) for $CD4^{-/-}$ mice immunized with the *M. tuberculosis* mutant strain.

Please amend the paragraph beginning on page 71, line 26, as follows:

Histologically, significant differences were observed between the vaccinated groups and naïve $CD4^{-/-}$ controls. The unvaccinated mice developed severe lung lesions with multiple large inflammatory nodules; some coalescing and spreading to areas of extensive diffuse pneumonia. The inflammatory response consisted of macrophages, numerous neutrophils, accompanied by low numbers of

lymphocytes. There were large numbers of acid-fast organisms in the lesions (FIG. ~~25a,d~~ 25A,D). In contrast, mice vaccinated with mc^{2}6030 (FIG. ~~25b,e~~ 25B,E) or BCG (FIG. ~~25e,f~~ 25C,F) showed reduced severity of the lung lesions. These mice showed scattered distinct lesions, adjacent to airways and localized, which remained smaller in diameter than in unvaccinated mice. These multifocal areas were composed of macrophages and numerous lymphocytes. There was a marked reduction in the numbers of acid-fast organisms compared to naïve controls.

Please amend the paragraph beginning on page 72, line 4, as follows:

The ability of mc^{2}6030-vaccinated CD4$^{-/-}$ mice to control the tuberculous challenge suggests the potential role of CD8$^+$ T cells in mediating this protection. In order to directly demonstrate the role of CD8$^+$ T cells in this protective response, CD4$^{-/-}$ mice that were vaccinated with mc^{2}6030 were treated with anti-CD8 monoclonal antibody and subsequently challenged with virulent *M. tuberculosis* through the aerosol route. Flow cytometric analysis showed that the anti-CD8 antibody treatment had depleted >99% of CD8$^+$ T cells from the lungs and peripheral blood. Surprisingly, repeated injections of the anti-CD8 antibody did not reduce the vaccine-mediated protective immune response. At 28 days post-aerosol challenge, the bacterial burdens in the lungs and spleens of the anti-CD8 antibody-treated immunized CD4$^{-/-}$ mice and nontreated vaccinated mice were similar; significant reductions in the pulmonary and splenic bacterial CFUs, relative to nonimmunized controls (>2 $\log_{10}$ in the lungs and >1 $\log_{10}$ in the spleen) were detected for each vaccine group (FIG. ~~24d,e~~ 24D,E).

Please amend the paragraph beginning on page 72, line 16, as follows:

To examine the role of IFN-γ in mediating the anti-tuberculous immunity evoked by the mutant *M. tuberculosis* strain, GKO mice were vaccinated with $mc^2$6030 or BCG-P and challenged with *M. tuberculosis*. Immunization of GKO mice with $mc^2$6030 or BCG-P did not significantly increase the survival period in comparison to unvaccinated controls with all mice in each of the three groups succumbing to the resulting infection within 30 days. Interestingly, 4 out of 10 BCG-vaccinated GKO mice died of disseminated BCG infection even before the aerosol challenge (FIG. ~~24f~~ 24F).

Please replace original Figures 1, 2, 3, 4, 5, 6, 18 and 25 with replacement Figures 1, 2, 3, 4, 5, 6, 18 and 25 attached hereto as **Exhibit 1**.